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13. ABSTRACT (Maximum 200 Words)

Stress fractures of bone constitute the most serious musculoskeletal overuse injury during military training of male and female recruits. We believe that the cascade of events in stress fractures is structured as the upregulation of bone turnover, amplification of porosity, induction of greater local strains and associated increase in damage activity a priori to fracture. The hypothesis of our study is that the onset of stress fractures can be predicted by monitoring the evolution of microdamage activity using acoustic emissions. During the first year bone tissue was procured and amassed for specimen preparation. A new mechanical loading set up was designed and fabricated to improve the cyclic loading tests. Histological protocols were developed for sequential labeling of bone tissue prior to and after mechanical loading to separate in vivo and in vitro microdamage. Acoustic emission tests were run on a preliminary group of samples and the characteristic of extraneous waveforms emanating from other sources were obtained for filtering purposes. In the overall the protocols have been finalized and the proposed tests will be accomplished during the second year.

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INTRODUCTION

The overall hypothesis driving this work was that an increase in microdamage activity during repeated loading of bone will signal the approaching stress fracture. Interception with the training regime prior to the incidence of the fracture as signaled by acoustic emissions would reduce the time necessary for recuperation and it would increase the preparedness and effectiveness of military personnel consequently. In order to test this hypothesis, fresh-frozen human tibias from males and females between the ages of 20 and 50 years old are being acquired for use in testing procedure outlined in the below. Sections of the tibias will be removed and machined into a proper geometry for fatigue tests. The formation and propagation of microdamage, in the form of microcracks and diffuse damage, produces detectable acoustic emission events similar to the seismic motions of an earthquake. Microdamage formation with characteristic waveform properties is being monitored through the use of piezoelectric acoustic emission transducers. Following fatigue testing, histological stains are used to label the microdamage present in the samples prior to and after fatigue testing to quantify the amount and morphology of the damage in order to develop relationships between the histological parameters and acoustic emission parameters. These relationships will be used to identify the acoustic emission parameters that are best able to predict the total score of damage.

BODY

There are three primary objectives in this study. During the first two years, it is planned to 1) determine the characteristics of damage events that signal the onset of stress fractures and 2) quantify the differences between genders on the evolution of fatigue damage. These two objectives will be accomplished concurrently during the first two years of the project. Attainment of these aims requires:

a) Preparation of specimens from male and female tibias
b) Mechanical induction of fatigue damage within these specimens through fatigue tests
c) Monitoring microcracks using acoustic emission technique in real-time during fatigue loading
d) Histological characterization of damage amount and damage morphology
e) Analysis of the data obtained from above tests.

The last primary objective of this work is to 3) investigate and quantify the damage events during the *in vitro* fatigue of whole bone stress fracture model. This third objective requires the completion of the first two aims and it is planned to proceed in the third year. The following will detail the progress made toward achieving the first two objectives during the first year of the study.

Procurement of Tibias and Specimen Preparation

Step a) requires the collection of male and female donor tibias from an age group relevant to military recruits. Since the focus of this work is the fatigue/fracture properties of those likely to be subjected to the rigors of military training, we needed to restrain our donor pool to a relevant age range. We initially specified an age range between 18 and 30 years old to various tissue collection agencies (NDRI, MTF, IIAM); however, the range was redefined as 18-55 years-old due to the limited number of donors available within the former age range.

Since September of 2003 we have received tibia to cover five males (aged 22, 22, 25, 36, 48 and 52 years old) and four females (25, 31, 46 and 49 years-old). Due to the limited number of specimen donors in our defined age range, this tissue acquisition step in the process appears as though it will take some time to acquire enough specimens to have a properly balanced testing sequence.

Mechanical Testing Hardware

We chose to use a four-point bending test configuration in order to induce the fatigue damage to the specimens due to this configuration's ability to subject the specimens to both tensile and compressive loading modes, which best reflects the complicated *in vivo* loads experienced by bones. The preliminary fatigue tests revealed

that some samples demonstrated an increasing stiffness with increasing number of loading cycles. Such a behavior is not a natural characteristic of bone's fatigue but it is the outcome of wear of the specimens at locations in contact with loading and support shafts (*1*). To resolve the wear issue a new four point bending set up was designed and built (Appendix A). The fixtures utilize shafts fixed to roller-bearing as the load bearing points. This allows the shafts to rotate freely as the specimen is loaded under bending. Specimen is fixed to one of the lower support shaft and moves freely along all other surfaces on Teflon films. Fixation of one surface was chosen because initially we observed specimens sliding laterally on the shafts during testing. In addition to the specimen loading fixtures, an environmental chamber in which the testing would take place was designed. Due to the length of time necessary to carry out the fatigue test (~ 5-14 hours), specimens are kept hydrated in a saline solution supplemented with $CaCl_2$ and protease inhibitors to prevent the degradation of the tissue's mechanical properties through mineral leaching and protein denaturation. The chamber is mounted to the testing apparatus and the lower loading fixture mounts inside the chamber. The chamber allows for the complete immersion of the specimen in solution heated to 37°C.

We plan to conduct the fatigue testing under load-controlled conditions and use the decrease in specimen compliance as a measure of test progress. In order to calculate the change in compliance more accurately a commercial displacement gage was modified with an extension which allowed the gage to be placed at the mid-span of the beam, rather than having to rely on the system's LVDT.

Monitoring Microcracks Using Acoustic Emission

The next step in the experimental setup was to setup the acoustic emission (AE) detection system. This system consists of two acoustic emission transducers, two signal amplifiers and a computer which analyzes and stores the AE data. The amplifiers, computer control board and software were acquired prior to the beginning of this study; new transducers that were small enough for the planned test configuration were acquired.

The transducers are mounted to the specimen using cyanoacrylate glue and held in place by two specially designed clamps. The transducers are mounted at the bounds of the inner mid-span region. In addition, through preliminary investigations, we have been able to isolate certain upper and lower limits for AE parameters indicative of crack formation and are therefore able to filter out any extraneous noise from the machine or motion of the fixtures and keep only the signals of interest.

Preliminary testing was conducted in order to check the test setup for determining the AE parameters during testing. Beam shaped cortical bone specimens (n = 4) were machined from the diaphyses of femurs such that the longer axis of the beams were aligned along the longer axis of the mid-diaphyseal shaft. Fatigue testing was conducted at four different stress levels and the AE parameters were recorded until the specimen failed. The test machine recorded the load and displacement data from the load cell and machine actuator displacement, and a pseudo-compliance measure was determined by dividing the difference in maximum and minimum displacement by the difference in the maximum and minimum load (Δ displacement / Δ load). We observed that the increase in the rate of acoustic emission events was concomitant with the characteristic knee region of the compliance curve which shoots up early in the cascade of events which leads to failure. Results of the this pilot study demonstrated that failure of standardized cortical bone specimens can be predicted ahead of time by monitoring the rate of damage accumulation via the acoustic emission technique. The predictive ability of the technique was such that the failure was detected within 78% ±15% of the fatigue life on the average. Please refer to the attached abstract submitted to the Annual Meeting of the Orthopaedic Research Society (Appendix B).

Histological Characterization of Damage

In our original proposal, we planned to use basic fuchsin to stain to label the microdamage and characterize the various histological parameters such as crack density, crack type and crack orientation of the tissue. While the use of basic fuchsin method for detecting and measuring crack properties has been the primary protocol used in the past, recent studies have concluded that an alternative has more to offer (*2-4*). The protocol for staining using basic fuchsin calls for dehydration of the specimen overnight in ethanol and embedding in polymethylmethacrylate (PMMA). The main problem with this technique is that there is no way to determine the difference between damage that existed prior to testing and the damage induced by testing. These problems can be overcome using two different aqueous chelating fluorochromes before and after testing the specimen to differentiate between the damage induced prior to (i.e. *in vivo*) and following (i.e. *in vitro*) mechanical loading. This method will allow each specimen as its own control in terms of identifying the baseline damage and will eliminate the need to prepare a control group for obtaining an average baseline damage value. Therefore we decided to amend our previous proposal of using basic fuchsin and perform our histological characterization of microdamage using other fluorescing agents.

We plan to stain the samples prior to testing with the agent that has the highest affinity for calcium (alizarin complexone) to label any preexisting microdamage. Following testing the agent with the second highest calcium affinity (calcein) will be used to stain the damage induced due to fatigue loading. The specimens will then be sectioned in both the longitudinal and transverse directions and the histological parameters determined. Each of these chelating agents fluoresces in different wavelength when exposed to epifluorescent light. Therefore, it will be possible to determine the crack growth rates of preexisting microdamage as well as the differences between preexisting, fatigue-induced, and embedding-induced microdamage (which will not be stained at all).

While verification of this method of microdamage labeling is provided in the literature (2-4), is has not yet been reported if the stains and the procedure for their application changes the mechanical properties of bone. The staining procedure specifies that the specimen be placed in a solution of the staining agent and then exposed to a vacuum, forcing the uptake of the stain into the tissue. While this occurs and very low vacuum pressures, we have conducted tests to determine if the mechanical properties are affected by the stain and/or the vacuum exposure. So far, we have tested samples under monotonic conditions and have found no significant differences ($p < 0.05$) in elastic modulus, fracture stress, yield stress, ultimate stress, fracture strain, yield strain, ultimate strain, resilience, or work to fracture related to the staining process. Data from these initial monotonic tests is available in Appendix C. We are currently in the process of conducting four point bending fatigue tests of specimens subjected to a similar treatment to test the effect of these stains on the fatigue performance of bone.

Analysis of Data Obtained

We have obtained data from the preliminary testing of 1) the test fixtures used to induce fatigue damage, 2) the acoustic emission system, and 3) a method of histological staining that will allow us to differentiate whether the microdamage was induced prior to or during fatigue testing. The data analysis methods used for each of these three are detailed below.

Four-point bending tests were initially run to verify the usability of the test system (i.e. test machine, loading regime, testing fixtures, and specimen hydration). Also, additional data was acquired during the preliminary testing of the acoustic event detection system. Using the computer that controls the test machine, the programmed load and displacement as well as the actual loads and displacements achieved by the test system

were recorded. This information is used to monitor the changing compliance of the test system. An Excel spreadsheet has been developed to use this output to calculate the changing compliance, as well as the stress and strain experienced by the specimen. As was expected, the compliance was seen to remain steady throughout most of the test followed by a dramatic increase before failure of the specimen (Appendix B, Figure 1). This occurred without regard for the stress range used for each specimen, although with higher stress ranges, failure occurred after a fewer number of cycles (Appendix B, Table 1). In further testing, an externally mounted displacement gage will provide an even more accurate data on the displacement and thus strain state of the specimen, although this gage has yet to be used in actual test.

The acoustic emission detection, as mentioned previously, has been used in preliminary tests. In the beginning, the software collected information on all acoustic events, regardless of whether they originated from microdamage or somewhere else. Eventually we were able to isolate the signals that did come from microdamage formation and were able to filter out unwanted signals based on their average frequency, duration, amplitude, and intensity. With the non-microdamage signals removed from the data, we were able to determine the number acoustic events related to bone damage as well as the time at which they occurred. Further analysis of these signals has not yet begun, although we plan to develop specially designed software to perform a more detailed cluster and principal component analysis to segregate acoustic emissions which belong to particular damage events.

Preparation and examination of samples stained with the two chelating fluorochromes (alizarin complexone and calcein) that we plan to use has been performed. Using fluorescence microscopic techniques we have been able to identify microdamage in cortical bone specimens (Figure 1). Monotonic tensile testing conducted thus far concerning the effect of using these stains would have on the mechanical properties of the bone have indicated that there is no effect (MANOVA followed by *post hoc* Mann-Whitney U-test, $p < 0.05$), although fatigue testing has not yet been completed.

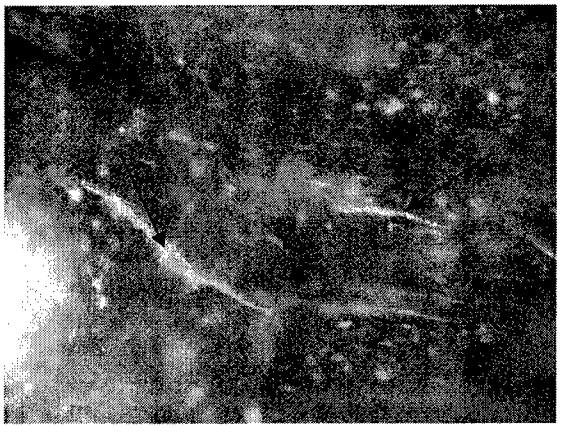

Figure 1: Photographs of bone taken near the fracture surface of specimens tested to failure in tension and stained with calcein. The arrows indicate the fluorescing microcracks.

KEY RESEARCH ACCOMPLISHMENTS

Performed preliminary tests to verify whether acoustic emissions signal the onset of failure well below the final fracture and the results are submitted to the Annual Meeting of the Orthopaedic Research Society as a conference abstract.

- Acquisition of 16 donor tibias (some paired, some not) from which four-point bending test specimens will be machined.

- Designed and built a four-point bending apparatus that:
    - Fixes the specimen at one point and uses free-rolling loading points to avoid specimen wear and undesirable lateral motion
    - Accommodates the specimen size needed for standardized testing
    - Allows the attachment of two miniature acoustic emission transducers
    - Allows specimen displacement monitoring from an external displacement gage
    - Allows complete immersion of the specimen in saline bath
- Obtained acoustic emission transducers and eliminated extraneous events which stem from activites other than the formation of microdamage.
- Established protocols to double label specimens before and after mechanical testing to detect microdamage using fluorescence microscopy.
- Conducted preliminary testing to determine the effect of double staining protocol on the monotonic mechanical properties of bone. Initial results indicate no change in mechanical properties of bone due to double staining procedure.

REPORTABLE OUTCOMES

Preliminary testing of our test setup, including the loading fixture setup, acoustic emission system setup, and verification of a more advantageous histological staining method. One preliminary investigation into the characterization of acoustic events during a fatigue test yielded and abstract that was submitted to the Orthopaedic Research Society's annual conference. The abstract is available in Appendix (C), but a brief summary will follow. Four samples of human cortical bone were fatigued at different stress levels in order to induce the formation of microdamage, which was then recorded by the acoustic emission system. We observed that 1) an increase in stress range decreases the fatigue life of the specimen, 2) the compliance, while steady through most of the test, has a sharp increase prior to failure, and 3) a dramatic increase in the number of acoustic emission events detected before the specimen fails. The increase in compliance is well correlated with the increase in acoustic events and it was therefore concluded that an increase in acoustic events can be used to predict the fatigue failure of bone. The results on the effects of fluorochrome stains on bone's mechanical properties are still being collected and we plan to present this work as a technical note for submission to the Journal of Biomechanics.

CONCLUSIONS

We set out with three primary objectives: 1) determine the characteristics of damage events that mark the onset of stress fractures and 2) determine the effect gender on the nature of fatigue damage in bone, and 3) investigate and quantify the damage events during an *in vitro* fatigue of whole bones. During this first year we focused on the first objective, which consisted of acquiring tibias from human donors, preparing them for testing, develop the test setup including the four-point bending fixtures, loading regimen, monitoring of the acoustic emissions in real-time during the fatigue test, and verification of histological examination techniques. It can be concluded from the work performed in this first year that we can use our four-point bending loading configuration to induce fatigue microdamage in bone and that we can monitor this microdamage from the acoustic events that its formation generates, and we can successfully use histological staining methods to examine the microdamage following the testing sequence.

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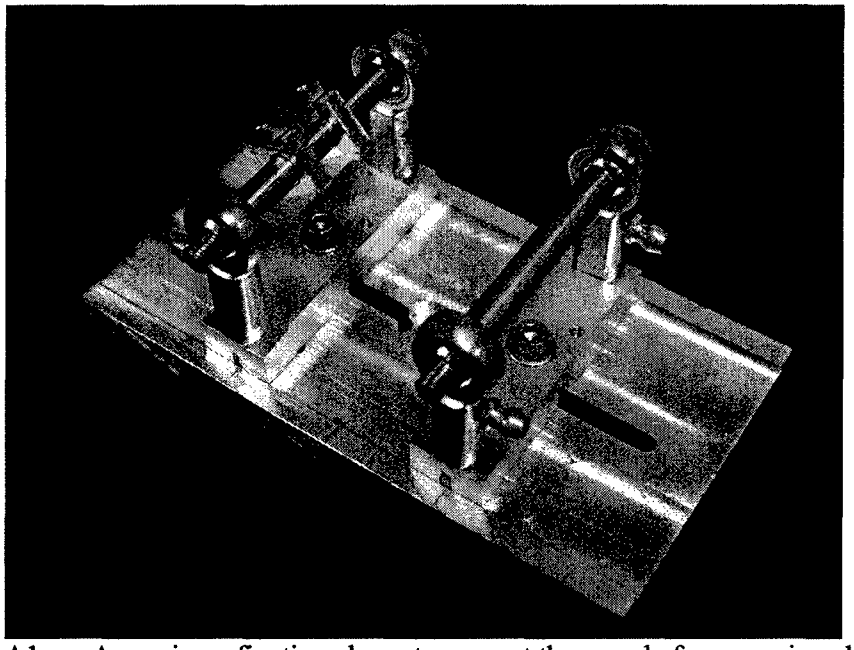
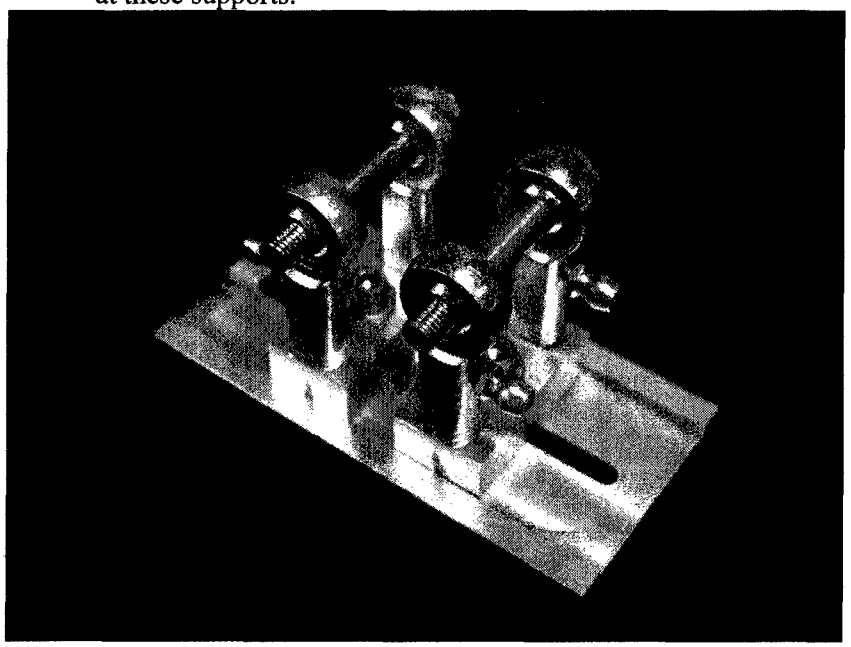
3. F. J. O'Brien, D. Taylor, T. C. Lee, *Journal of Biomechanics* **35**, 523-6 (Apr, 2002).
4. F. J. O'Brien, D. Taylor, T. C. Lee, *Journal of Biomechanics* **36**, 973-80 (Jul, 2003).

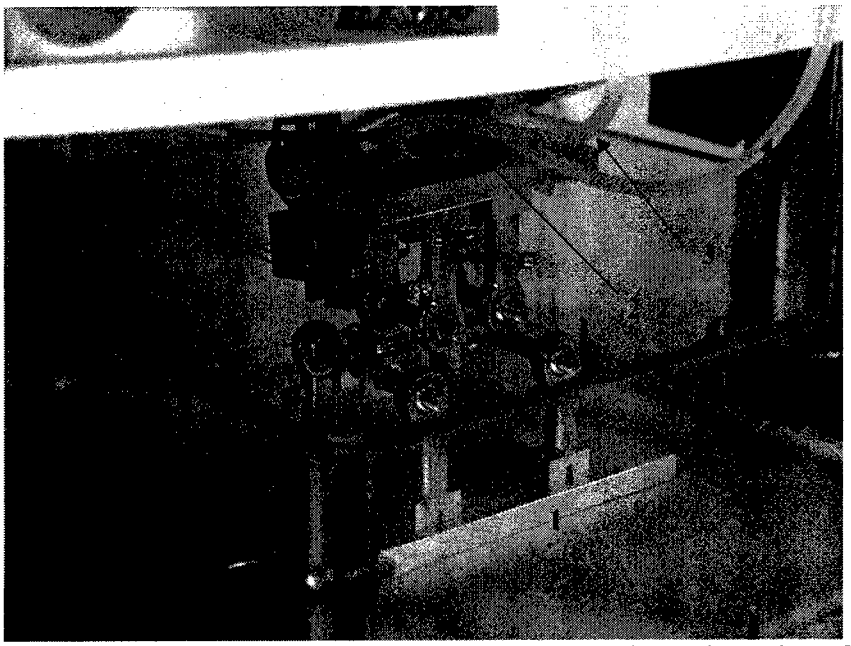
APPENDIX A: **Fixtures Used for Mechanical Induction of Damage (Four-point Bending)**



A1: A specimen fixation clamp to prevent the sample from moving along supports during testing.
A2: Free-rotating supports to apply the outer two loads of the four-point loading and prevent specimen wear at these supports.



B1: Free-rotating supports to apply the inner two loads of the four-point loading and prevent specimen wear at these supports.

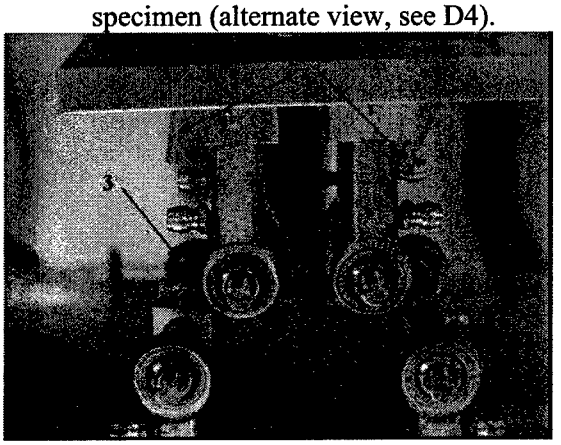


C1: Chamber that can be used for either a) complete submersion of the test in solution or b) to contain the solution that drips onto the samples to keep them moist (See C3).

C2: Submersible load cell (100 lb.) that will be used to measure the loads the specimens are subjected to.

C3: Tubes to carry solution from a container (not shown) and drip solution onto samples to maintain hydration (alternate view, see D1).

C4: Displacement gage to measure the specimen displacement for calculation of the strain state of the specimen (alternate view, see D4).



D1: Tubes to carry solution from a container (not shown) and drip solution onto the specimen (D2) to maintain hydration.

D2: Bone specimen mounted onto testing apparatus. Specimen dimensions are approximately 80 mm x 3 mm x 3 mm.

D3: Specimen fixation clamp to prevent to movement of the sample along the supports.

D4: Displacement gage measuring the specimen displacement.

APPENDIX B: **Abstracted submitted to the Orthopaedic Research Society's Annual Conference (2005)**

ACOUSTIC EMISSION TECHNIQUE CAN PREDICT THE FATIGUE FAILURE OF CORTICAL BONE

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INTRODUCTION

Stress fractures of bone constitute the most serious musculoskeletal overuse injury during military and athletic training (1). The cascade of events prior to the incidence of stress fractures involves the upregulation of bone turnover, amplification of porosity, induction of greater local strains and associated increase in damage activity. Therefore, we propose that it would be possible to diagnose the progression of the failure process by monitoring the microdamage activity. Acoustic emission technique detects the stress waves generated during the formation of microdamage using surface mounted piezoelectric transducers; thus, it is a viable alternative to monitor damage activity (2). More importantly, it is a non destructive and non invasive technique which has the potential to be utilized as an *in vivo* diagnostic tool. In the current study we sought to answer the following questions: 1) can the onset of stress fractures be predicted by monitoring the microdamage activity via acoustic emissions and 2) how soon before the actual failure can be predicted via acoustic emission. This hypothesis was investigated by listening to acoustic emissions released during in vitro fatigue loading of standardized specimens from human femoral cortical bone.

METHODS

Four prismatic beams were machined from the mid- femoral diaphyses of two males (52 and 53 years-old) using a low-speed saw (South Bay Tech). The widths and the thicknesses of beams were machined as allowed by the actual cross-sectional geometry of femurs, resulting in a thickness range of 2.5 mm to 3.3 mm and a width range of 6.5 mm to 7.5 mm. Lengths of beams were kept standard at 70 mm in length. Beams were subjected to fatigue in four point bending with an inner span length of 30 mm and outer span length of 60 mm under continuous irrigation of calcium supplemented saline solution (3). The cyclic loading was conducted under load control and the maximum load varied to create stresses corresponding to 60% to 80% of the yield stress which was obtained from prior monotonic tests of two prismatic beams. The minimum load was kept at $1/10^{th}$ of the maximum load. The loading waveform was triangular with a 0.1 sec long ramp down followed by a 0.1 sec long ramp up at the rate of 2 Hz. The manifestation of damaging events were assessed by calculating the compliance of specimens by dividing the strain range with the stress range as calculated from the load and displacement (as recorded at the loading point) values, respectively.

An acoustic emission transducer (Pico, PAC, NJ) was mounted at the mid-span of the specimens using cyanoacrylate glue. Signal from the transducer was preamplified and acquired at a rate of 2 MHz using a specialized acoustic emission system (AEDSP 32/16, PAC, NJ). The activity of microdamage was assessed by recording the cumulative number of acoustic emission events.

RESULTS

The specimens were loaded in the low-cycle fatigue regime and the number of cycles to failure (N_F) ranged from 600 to 26363 cycles (Table 1). Compliance curves exhibited two temporal stages: a region of stability where the compliance did not change notably and a second stage characterized by a knee region during which the compliance increased rapidly and concluded with failure of the specimen (Figure 1). Concomitant with the initiation of the knee region was an abrupt increase in the cumulative number of acoustic emission events indicating that prefailure events are predominantly highlighted by the microdamage activity.

We have taken the number of cycles at which the rate of accumulation of acoustic emissions begins (N_P) to spike as the cycle at which acoustic emissions predict the onset of failure. The capacity of acoustic emissions to predict failure was calculated in terms of the percentage of specimen's fatigue life, i.e. $(N_P/N_F)*100$. In all specimens precursor acoustic emission events were detected in the range prior to the characteristic knee region and these events were likely to stem from initiation of microdamage.

DISCUSSION

Results of the current study demonstrated that failure of standardized cortical bone specimens can be predicted ahead of time by monitoring the rate of damage accumulation via the acoustic emission technique. The predictive ability of the technique was such that the failure was detected within 78% ±15% of the fatigue life on the average. The current analysis has focused on the rate of accumulation of acoustic emissions only. Acoustic emissions are sinusoidal bursts and further valuable information could be extracted from these bursts such as the duration, amplitude, energy and the frequency content. These waveforms can be classified to identify and extract those bursts which mark the onset of failure (4). Further refinement of the method holds promise for *in vivo* detection of stress fractures in the field using acoustic emissions.

Table 1. The predictive capability of acoustic emissions expressed in terms of the specimen's fatigue life

	Maximum Stress [MPa]	N_F, Fatigue Life [cycles]	N_P, Fracture Onset via AE [cycles]	Predictive Capability [% of Fatigue Life]
Specimen 1	55	26363	22580	85%
Specimen 2	61	35020	33382	95%
Specimen 3	66	4737	2989	63%
Specimen 4	71	600	402	67%

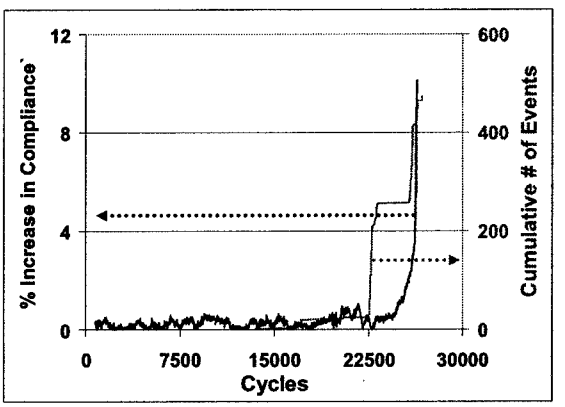

Figure 1. The variation of compliance and the cumulative number of acoustic emission hits with loading cycles.

ACKNOWLEDGEMENTS: This study was funded by the U.S. Army Medical Research and Materiel Command. Tissue was provided, in part, by the Musculoskeletal Transplant Foundation.

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APPENDIX C:

Treatment	Yield Strain (με)	Ultimate Stress (MPa)	Fracture Stress (MPa)	Yield Stress (MPa)	Modulus of Elasticity (GPa)	Work to Fracture
Ca-Supplemented Solution	0.0072 ± 0.0005	91.63 ± 13.17	90.88 ± 13.35	85.73 ± 10.38	16.65 ± 1.30	1.34 ± 0.72
Distilled H_2O	0.0072 ± 0.0003	94.58 ± 14.23	92.88 ± 15.16	87.61 ± 9.99	17.01 ± 1.90	1.26 ± 0.51
Alizarin	0.0070 ± 0.0004	92.53 ± 13.98	90.28 ± 12.00	86.80 ± 10.05	17.50 ± 1.86	1.29 ± 0.58
Calcein	0.0070 ± 0.0003	92.33 ± 17.26	90.25 ± 18.47	85.86 ± 12.06	17.32 ± 1.96	1.44 ± 0.82
Significance	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$